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Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens*, using isopentenyladenine

R. Reski and W.O. Abel
Institut für Allgemeine Botanik, Ohnhorststrasse 18, D-2000 Hamburg 52, Federal Republic of Germany

Abstract. The bud-inducing effect of the cytokinin N^6-(Δ^2-isopentenyl)-adenine (i^6-Ade) was examined in the moss *Physcomitrella patens* growing in liquid culture. Under these conditions, buds could be induced on chloronemata as well as on caulonemata. By application of i^6-Ade, bud-formation was accelerated in both types of tissue. The number of buds, their size and their site of development were dependent on the concentration of the cytokinin in the range of 10^{-7} M to 10^{-5} M. Moreover, the percentage of caulonema cells increased with a cytokinin concentration of 10^{-5} M. These results indicate that chloronema cells may also function as target cells for exogenous cytokinins. The composition of proteins from caulonemata and chloronemata of two different species (*P. patens* and *Funaria hygrometrica*), grown on solid medium were compared. No differences could be detected between the protein patterns of caulonemata and chloronemata of the same species while between the two species the differences were obvious.

Key words: Bryophyta – Bud induction (mosses) – Caulonema, specific proteins – Cytokinin and bud induction (mosses) – Differentiation (moss) – *Funaria* – *Physcomitrella*.

Introduction

Cytokinins can increase gametophore production in several mosses (Bopp 1963; Szweykowska 1963; Iwasa 1965; Brandes 1967; Hahn and Bopp 1968), and Hahn and Bopp (1968) have employed budding as a specific test for exogenous cytokinins. According to Bopp (1974) only caulonemata contain target cells for exogenous cytokinins, a view supported by the detection of caulonema-specific proteins (molecular weight approx. $5 \cdot 10^5$ dalton (Da) in *Funaria hygrometrica* using an one-dimensional native electrophoresis. These caulonema-specific proteins were shown to bind exogenous cytokinins (Erichsen et al. 1977). Sood et al. (1978) also observed caulonema-specific proteins in different moss species.

So far, relatively little attention has been given to reports about budding on chloronema cells. Nehlsen (1979) reported on budding which occurred shortly after spore germination, and Sood and Chopra (1973) described budding which occurred directly out of the spore.

We now describe experiments which show several concentration-dependent effects of the native cytokinin isopentenyladenine (Beutelmann and Bauer 1977) on moss protonemata in liquid culture, indicating that chloronema cells may also function as target cells for exogenous cytokinins. In addition, attempts were made to identify caulonema-specific proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver-staining techniques.

Abbreviations: i^6-Ade = N^6-(Δ^2-isopentenyladenine); Da = dalton; SDS-PAGE = sodium dodecyl sulfate – polyacrylamide gel electrophoresis

Materials and methods

Nutrition salts. A modified Knop medium was used (Bopp and Brandes 1964): KH_2PO_4 (250 mg l^{-1}), KCl (250 mg l^{-1}), $MgSO_4 \cdot 7\,H_2O$ (250 mg l^{-1}), $Ca(NO_3)_2 \cdot 4\,H_2O$ (1000 mg l^{-1}), $FeSO_4 \cdot 7\,H_2O$ (12.5 mg l^{-1}). In addition, the pH was adjusted to 5.8 using 1 N NaOH.

Liquid culture. The protonemata were raised under sterile conditions in 50-ml Erlenmeyer flasks containing 20 ml of culture medium and covered with silicone sponges (Bellco, Vineland, N.J., USA). The protonemata were cultured in a growth chamber at $24 \pm 1°$ C with laterally incident light provided by eight fluorescent tubes (Philips TLF 40 W/33) giving a light flux of 9000 ± 1000 lx (outside the flasks). The light-dark regime

was 16:8 h. Cultures were shaken on a GFL shaker (Hannover, FRG; type 3015) set at 100 rpm.

Protonemata were raised from regenerating moss fragments: mosses in liquid culture were disintegrated at 20000 rpm by an Ultra-Turrax (type 18/10; IKA, Staufen, FRG). The resulting suspension contained moss fragments of 5–10 cells. The culture flasks were inoculated with aliquots of 50 µl, giving about 20 protonemata per flask.

Twelve days after inoculation the first caulonema cells could be observed, and the culture medium was adjusted to 10^{-7} M, 10^{-6} M and 10^{-5} M (i^6-Ade). Three days later protonemata were photographed, buds were counted, chlorophyll was extracted using methanol and total protein was calculated using Serva Blau G (Bradford 1976) as a stain.

Solid culture. In order to detect caulonema-specific proteins, protonemata were raised either from single spores or from moss fragments on the described medium solidified with 1% Oxoid agar on cellophane (Bopp et al. 1964) in plastic Petri dishes. They were cultured in a growth chamber at 25±1° C with light from above from two Osram L 65 W/25 fluorescent tubes (Osram, Augsburg, FRG) giving 4000 lx light flux (on top of the Petri dishes) for 16 h a day. After 18–20 d caulonemata were observed, but no buds could be seen. Protonemata were separated under a stereomicroscope using a scalpel into a small central part (chloronema exclusively) and a peripheral part (caulonema and some chloronema).

Protein patterns of both types of tissues from *Physcomitrella patens* and *Funaria hygrometrica* were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Sodium dodecyl sulfate PAGE and silver staining. Either continuous or discontinuous systems were used according to Laemmli and Favre (1973). Gels were either 1.5 mm or 0.75 mm thick, the stacking gel being 4.5% polyacrylamide, and the running gel either 7.5% or 10% polyacrylamide. Silver staining of the protein patterns was carried out according to Morrissey (1981).

Results

Table 1 summarizes the effects of exogenous i^6-Ade on the differentiation of *Physcomitrella* protonemata. After 15 d in the medium without i^6-Ade (control) no buds were present. The media containing 10^{-7}, 10^{-6} and 10^{-5} M i^6-Ade induced 51±8.6, 40±4.8 and 150±15.6 buds per µg protein, respectively (Table 1; Figs. 1, 2, 3). Buds in 10^{-6} M i^6-Ade were larger than in 10^{-7} M i^6-Ade; in 10^{-5} M the buds were the smallest (Table 1; Figs. 1, 2, 3). Isopentenyladenine at concentrations of 10^{-7} M and 10^{-6} M induced buds on chloronema cells whereas at 10^{-5} M i^6-Ade buds were induced on caulonema cells (Table 1; Figs. 1, 2, 3). The percentage of caulonema cells in the control medium and in the media containing 10^{-7} and 10^{-6} M i^6-Ade was nearly the same, but in 10^{-5} M i^6-Ade the number of caulonema cells evidently increased (Table 1; Figs. 1, 2, 3).

In liquid medium supplied with 10^{-6} M i^6-Ade, budding also occurred on chloronema cells of *F. hygrometrica* (Figs. 4, 5).

Table 1. Effects of isopentenyladenine on the differentiation of *Physcomitrella patens* protonemata

	Concentration of i^6-Ade			
	Control	10^{-7} M	10^{-6} M	10^{-5} M
Number of buds per µg total protein (mean±SE)	0	51±8.6	40±4.8	150±15.6
Size of buds (state of differentiation)[a]	–	++	+++	+
Buds developing at	–	Chloronema	Chloronema	Caulonema
Estimated percentage of caulonema cells	5–15%	5–15%	5–15%	30–40%

[a] –, no buds developed; +, small buds, most of them three cells; ++, buds with about 10 cells; +++, large buds, some leaves developed

The composition of proteins from caulonemata and chloronemata of *P. patens* and *F. hygrometrica* (grown on solid medium) was analysed by SDS-PAGE. Proteins from 12- and 21-d-old protonemata of *P. patens* grown in liquid medium were also analysed. No difference between the protein patterns of caulonemata and chloronemata of the same species could be detected, while differences between the two species and between the *P. patens* protonemata of different ages were obvious. In comparison with *P. patens*, three additional proteins were found in *F. hygrometrica* (27000, 21000 and 20000 Da) (Fig. 6; lanes 1, 2, 5, 6). In comparison with 21-d-old *P. patens* protonemata, there was one additional protein of approx. 46000 Da in 12-d-old *P. patens* protonemata (Fig. 6; lanes 3, 4; for all protein patterns see Fig. 6).

Discussion

Cultures in shaken liquid medium were shown to be suitable for examining the effects of exogenous substances on *P. patens*. Even in the long run, this culture technique allowed normal protonemata differentiation, including the production of leafy gametophores. Furthermore, the agitated liquid medium ensured an even distribution of exogenously applied substances accessible to every cell of a protonema.

At the stage of development examined in our experiments, the chloronema cells particularly seem to have the capability to react to exogenous cytokinins. We assume that there are two possible

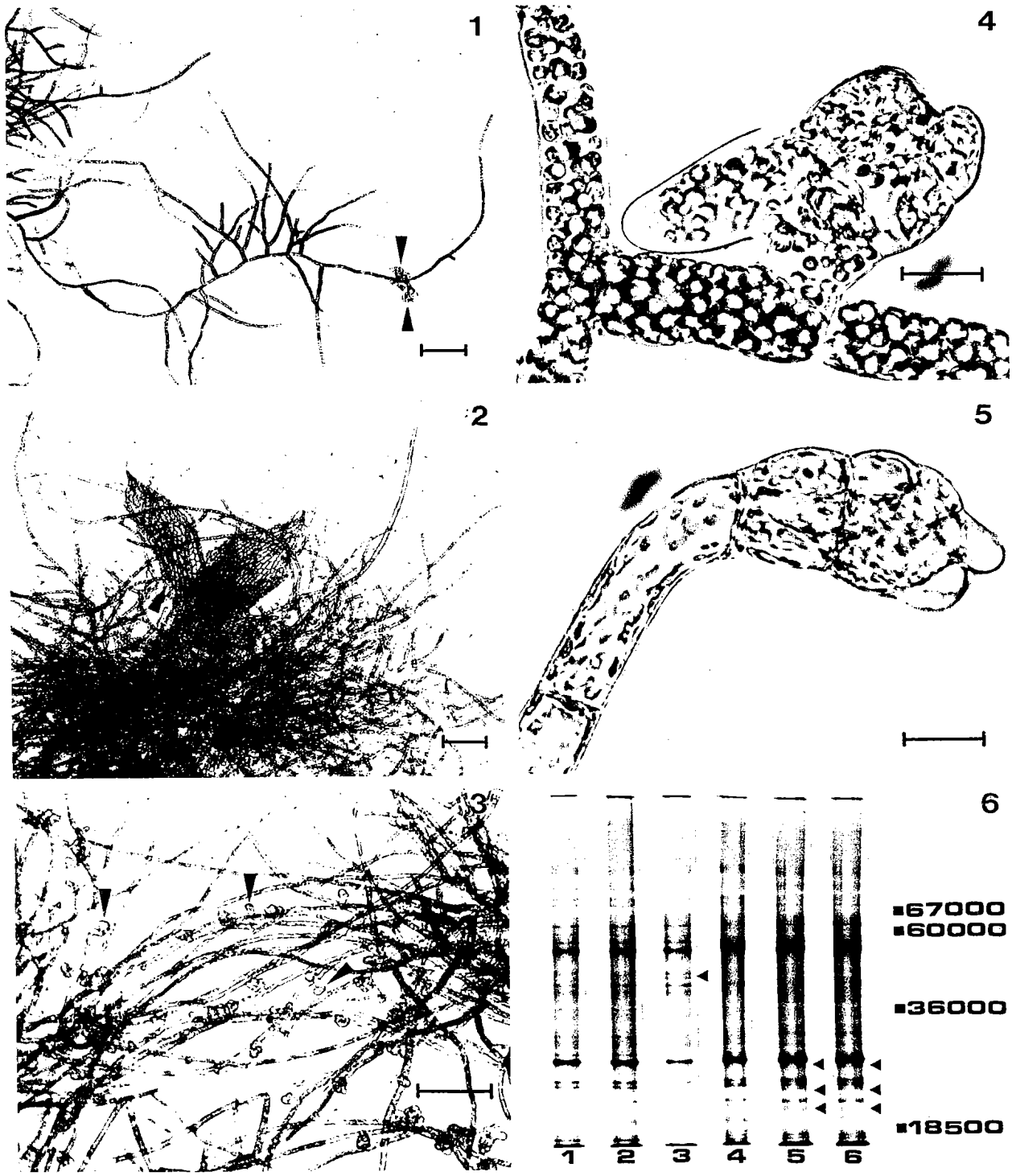

Fig. 1. *Physcomitrella* protonema (15 d old) after 3 d in 10^{-7} M i^6-Ade, showing budding (►) on chloronema cells. Bar = 0.5 mm. **Fig. 2.** *Physcomitrella* protonema (15 d old) after 3 d in 10^{-6} M i^6-Ade, showing large buds and leafy gametophores (►). Bar = 0.5 mm. **Fig. 3.** *Physcomitrella* protonema (15 d old) after 3 d in 10^{-5} M i^6-Ade, showing an increased percentage of caulonema cells and a large number of very small buds developing on caulonema cells (►). Bar = 0.5 mm. **Fig. 4.** Bud on chloronema cell of *Physcomitrella patens* induced by 10^{-6} M i^6-Ade. Bar = 50 µm. **Fig. 5.** Bud on chloronema cell of *Funaria hygrometrica* induced by 10^{-6} M i^6-Ade. Bar = 50 µm. **Fig. 6.** 10% slab-gel from discontinuous SDS-PAGE. *Lane 1, Physcomitrella* chloronema on solid medium, 18 d old; *lane 2, Physcomitrella* caulonema on solid medium, 18 d old; *lane 3, Physcomitrella* protonema in liquid medium, 12 d old. Additional protein of approx. 46000 Da (►); *lane 4, Physcomitrella* protonema in liquid medium 21 d old. No additional protein after that time; *lane 5, Funaria* chloronema on solid medium, 20 d old; *lane 6, Funaria caulonema* on solid medium, 20 d old; *lanes 5, 6, Funaria* has additional proteins of approx. 27000, 21000 and 20000 Da (►)

steps of development, depending on the amount of cytokinin available. Up to a certain limit, chloronema cells react by budding (10^{-7} M and 10^{-6} M). Buds induced by 10^{-6} M i^6-Ade were larger at the time of comparison than those induced by 10^{-7} i^6-Ade, because of earlier induction (daily controls after adding cytokinin) and perhaps faster growth. When the concentration of the cytokinin is raised above that limit to 10^{-5} M i^6-Ade, however, chloronemata do not react by budding but by forming caulonemata. Afterwards, buds develop on caulonema cells.

The cytokinin-influenced differentiation we observed may be a result of the culture methods applied here. Experiments showing budding on caulonema cells only were usually carried out on solid medium (Bopp 1974). Protonemata growing on agar-solidified growth media will produce concentration gradients around them; moreover, not all of the cells come into contact with the substances applied. Consequently, it is difficult to compare our results with earlier experiments, especially with regard to the effective cytokinin concentrations. Furthermore, most of the cytokinin effects reported hitherto were induced by the artificial cytokinin kinetin (Bopp 1974; Bopp 1982), which has effects on moss morphogenesis different from those of native cytokinins (Bopp 1982). Therefore, we chose the cytokinin i^6-Ade which is accepted as a native cytokinin in *F. hygrometrica* and *P. patens* (Bopp 1982).

Until now, the capability of chloronema cells to react to cytokinins by budding was known only for *Polytrichum juniperinum* (Nehlsen 1979) and *Entodon myurus* (Sood and Chopra 1973) but was not described for *F. hygrometrica* or *P. patens* (Bopp 1981). According to Bopp (1974) only caulonemata contain target cells for exogenous cytokinins, a suggestion supported by the detection of caulonema-specific proteins (CSP; molecular weight approx. $5 \cdot 10^5$ Da) in *F. hygrometrica*. Moreover, these proteins were shown to bind exogenous cytokinins in vitro (Erichsen et al. 1977). Sood et al. (1978) observed CSP in different moss species, e.g. in two mutants of *P. patens* which do not form buds (or at most very few) without exogenous cytokinins (Ashton and Cove 1977).

Erichsen et al. (1977) and Sood et al. (1978) both used one-dimensional gradient gel microelectrophoresis to demonstrate the existence of CSP. But Erichsen et al. (1977) also reported that CSP subunits could be detected by SDS-PAGE without presenting any experimental data for this statement. We, therefore, used the more powerful slab-gel-electrophoresis (SDS-PAGE) to examine the existence of CSP on *F. hygrometrica* and *P. patens*. Although our separating methods were more powerful and although silver staining is far more sensitive than the traditional staining with Coomasie Blue or amido black used by Erichsen et al. (1977) and Sood et al. (1978), we were unable to detect different protein patterns in caulonemata and chloronemata either in *F. hygrometrica* or in *P. patens*. Moreover, the CSP are not apparently reproducible by Bopp and coworkers themselves (Prof. M. Bopp, Botanical Institute, University of Heidelberg, FRG; personal communication).

Ashton et al. (1979a, b) reported concentration-dependent effects of cytokinins and auxins on gametophytic growth and development in *P. patens* using auxin- and cytokinin-resistant mutants. Our experiments demonstrate that different concentrations of the cytokinin i^6-Ade may have different qualitative effects on protonema differentiation, showing that the role of cytokinins may be more complex than was previously suspected.

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